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The immunological role of B7-H4 in pregnant women with Sars-Cov2 infection

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Abstract

Problem: T-cells are key players in fighting the coronavirus disease 2019 (COVID-19). The checkpoint molecule B7-H4, a member of the B7 family, can inhibit T-cell activation and proliferation by inhibiting NF-kb expression. We aimed to elucidate the immunological role of soluble B7-H4 (sB7-H4) and B7-H4 in pregnant women suffered from an acute Sars-Cov2 infection.

Methods: Expression levels of sB7-H4 and cytokines were detected by enzyme linked immunosorbent assay. B7-H4 and cytokines mRNA expression was analyzed by qPCR, and B7-H4 and NF-κb (p65) protein levels were investigated by western blot and immunofluorescence staining in placenta chorionic villous and decidual basalis tissues of COVID-19 affected women and healthy controls.

Results: Fibrinoid necrosis in the periphery of placental villi was increased in the COVID-19-affected patients. sB7-H4 protein in maternal and cord blood serum and IL-6/IL-10 were increased while leukocytes were decreased during SARS-CoV-2 infection. Serum sB7-H4 level was increased according to the severity of SARS-Cov-2 infection. Cytokines (IL-6, IL-18, IL-1β, TNF-α), B7-H4 mRNA and protein in the decidual basalis tissues of COVID-19-infected pregnant women were significantly increased compared to healthy controls. IL-18 and IL-1β were significantly increased in the placenta chorionic villous samples of COVID-19 affected patients, while NF-κb (p65) expression was decreased.

Conclusions: The expression of the immunological marker sB7-H4 correlated with the severity of COVID-19 disease in pregnant women. sB7-H4 and B7-H4 can be used to monitor the progression of COVID-19 infection during pregnancy, and for evaluating of the maternal immune status.

KEYWORDS
B7-H4, immune regulation, infection, pregnancy, SARS-CoV-2 T-cells

Liyan Duan, Beatrix Reisch, Alexandra Gellhaus and Antonella Iannaccone contributed equally.

1 | INTRODUCTION

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) leading to the coronavirus disease 2019 (COVID-19 disease) has upset the world since early March 2020.[1] Also at the same time, the World Health Organization (WHO) had announced SARS-CoV-2 a pandemic. During this period, pregnant women have received additional attention, as a result of special immune status, which need to adapt to make sure the tolerance to the fetus, at the same time preserving immune protection.[2] The immune system plays a central role in mediating a successful pregnancy. Following infection with SARS-COV-2, an initial innate immune response occurs, leading to activation of the adaptive immune system and differentiation of T-cells, including the production of cytokines of various T-cell subsets. The virus can be killed by CD4+T cells through B lymphocytes with specific antibodies against the virus, while virus infected cells can be killed by cytotoxic CD8+ T cells.[3] This is essential to balance the immune system's reaction to pathogens and heightened inflammation during antiviral processes.[4] Neutrophils, lymphocytes and monocytes are recruited to the site of infection by pro-inflammatory cytokines and chemokines, leading to an elevated inflammation response.[5] Maternal inflammatory responses in the circulation are important for successful development of the embryo in the uterus. Fetal trophoblast (syncytiotrophoblast (STB)), extravillous trophoblast (EVT) cells and decidual immune cells play an important role in the interaction of the maternal-fetal interface in SARS-CoV-2 infection during pregnancy.[6,7]

Previous studies have shown that pregnancy did not seem to increase the risk of SARS-CoV-2 infection compared with non-pregnant women, but pregnant women with severe COVID-19 disease are at higher risk: they are more likely to be admitted to the intensive care unit (ICU), receive invasive ventilation and extracorporeal membrane oxygenation and die.[8,9] For pregnant women infected with SARS-CoV-2, complications during pregnancy, such as preeclampsia, preterm birth and even stillbirths are increased compared to uninfected pregnant women.[10–13] Previous studies reported that plasma levels of TNF-α, IL-6, Il-8, IL-10, IL-1β, IL-18, IL-17A, IL-12, chemotactic cytokines (monocyte chemoattractant protein (MCP)-1), interferon gamma/IFNγ) and growth factors are elevated in COVID-19 –affected pregnant women and correlated with disease severity.[14–16] The excessive inflammation is an important element of severe COVID-19, under the umbrella term "cytokine storm," which is related to critical illness and high mortality of patients.[17] A review has reported that lymphopenia, elevated selected pro-inflammatory cytokines and elevated neutrophils are characteristic of both Sars-Cov-2 positive patients and pregnant women, thus concluding that pregnancy is a risk factor for progression of COVID-19 .[18]

Co-signalling molecules including co-stimulatory and co-inhibitory receptors are glycoproteins on cell surface that can modulate T-cell receptor (TCR) signals. With their co-stimulatory or co-inhibitory functions they can promote respectively suppress T-cell activation and therefore are important for inducing effective immune responses and preventing unwanted autoimmunity.[19] The B7 family can generate co-stimulatory and -inhibitory signals through separate interactions. B7-H4 (also named as VTCN1 or B7x or B7S1) has been recognized as a co-inhibitory member of the B7 family.[20–22] B7-H4 exists as both, a membrane-bound (B7-H4) and a soluble (sB7-H4) isoform. The membrane-bound form B7-H4 has the ability to suppress the proliferation of CD4+ T- and CD8+ T-cells.[23,24] Previous studies revealed that B7-H4 can inhibit T-cell activation by inducing cell cycle arrest and inhibiting NF-kb nuclear translocation.[25] Another study found that B7-H4 is also expressed on macrophages of placenta decidual basalis tissue.[26] We also found that B7-H4 was detected on both the STB and EVT.[27] Neither the origin nor the function of sB7-H4 is fully understood. However, it is known that sB7-H4 was secreted when cells were under inflammatory conditions.[28] Azuma et al. studied a mouse model of rheumatoid arthritis revealing that sB7-H4 can lead to the exacerbation of collagen-induced arthritis by acting as a decoy molecule to prevent the suppressive function of B7-H4 on the cell-surface, accompanied by enhanced T- and B-cells along with elevated activity of neutrophils.[29] However, there is more and more evidence that sB7-H4 serves as a T-cell-negative regulator that suppresses host immunity and allows tumor cells to evade immunological surveillance.[30–33]

Therefore, we here plan to identify the immunological role of sB7-H4 in maternal blood serum and cord serum from pregnant women suffered from COVID-19 at term compared to non-COVID-19-affected pregnant women. We also determined B7-H4 mRNA and protein expression in placental tissues in third trimester of women with and without COVID-19.

2 | MATERIALS AND METHODS

2.1 | Study design and participant enrollment

We took blood sera from pregnant patients in the third trimester (35+6 – 40+0 weeks of gestation). Blood from SARS-CoV-2 positive (for sB7-H4 analysis: $n = 17$; for cytokine analysis: $n = 21$) and SARS-CoV-2 negative control group (for sB7-H4 analysis: $n = 18$; for cytokine analysis: $n = 15$) were collected before spontaneous onset of labor or from women undergoing elective caesarean section. Cord blood was collected directly at delivery (COVID-19 pregnant women $n = 8$, control $n = 8$).

For placental tissues, 16 pregnant women (eight patients with acute SARS-CoV-2 infection in the third trimester of pregnancy, eight non-infected control pregnant women) were enrolled in this study from the department of Gynecology and Obstetrics at the University Hospital Essen, between 2020 and April 2021. These patients were diagnosed with SARS-Cov-2 infection by nasopharyngeal RT-PCR at the Institute of Virology at our hospital. According to WHO COVID-19 severity categorization, pregnant patients were divided into an asymptomatic/mildly or severely symptomatic group.[34] The exclusion criteria for the control group were the following: other pregnancy complications such as chronic villitis, chorioamnionitis or underlying autoimmune problems. All newborns in our study were tested negative for SARS-CoV2.

2.2 | Human placenta sample collection and processing

We prepared tissues from placental chorionic villous and decidua basalis using the same method as described in our previous publication.[27] Samples were taken at the time of vaginal delivery or caesarean section.

We stored all samples at −80°C until we use placenta chorionic villous and decidua basalis tissues to isolate RNA and protein (see details in Duan et al. 2021).[27]

We prepared the paraffin-embedded sections by excising the placenta chorionic villous tissue from the maternal part including the decidua for subsequent histological examination and immunofluorescence experiments.

2.3 | Enzyme-Linked immunosorbent assay (ELISA)

We used the ELISA Kit for V-Set Domain-Containing T-Cell Activation Inhibitor 1 (VTCN1 = sB7-H4) (Cloud-Clone Corp. #L211008816) to analyse the expression of sB7-H4 in maternal serum and blood cord according to the instruction manual and it is also found in our previously published article.[35] Shortly, for measurement of sB7-H4 levels by an absorbance at 450 and 620 nm were done using the ELISA reader (TECAN, Model Sunrise; Austria GmbH, Grodig, Austria) and the data analysis software Magellan™ (TECAN, Mannedorf, Switzerland). For quantification of sB7-H4 serum levels (ng/ml according to the established standard curve (detection range: 0.156–10 ng/ml), a non-linear regression model was utilized with a log/lin type of graph according to the manufacturer's instructions. The absorbance was directly proportional to the concentration level of sB7-H4 in the samples, which was calculated from the calibration curve. The minimum detection limit of sB7-H4 was typically <0.056 ng/ml. Intra-assay variation was <10%, while inter-assay variation was <12%.

2.4 | Multiplex assay

LegendPlex™ bead-based immunoassay (Biolegend, San Diego, USA, Cat. No. 740809) was used to measure the secretion of cytokines in maternal blood sera (TNF-α, IL-17A, IL-10, IL-6,IL-18, MCP-1). The protocol was performed regarding the manufacturer's recommendations. Briefly, we sonicated the detection beads for 1 min and then incubated the detection beads with diluted serum samples (1:2) or standard solution in a V-bottom microplate for 18 h on a plate shaker at 8°C. After centrifugation at 600 rcf for 5 min at room temperature, beads bound to target cytokines were washed and afterwards incubated with biontinylated detection antibodies for 1 h on a plate shaker at room temperature. We then added Streptavidin-Phycoerythrin directly to each well and incubated on a plate shaker for 30 min at room temperature in the dark. Centrifugation of the V-bottom microplate was done at 400 rcf for 5 min at room temperature. Separation of analyte-specific population and quantification of PE fluorescence signals is based on the principle that beads are differentiated by size and internal fluorescence intensity on a flow cytometer. We determined the concentration of a specific analyte using a standard curve generated in the same assay applying a 5-parameter curve fitting algorithm. Cytexpert 2.3 was used for data acquisition and accompanying LegendPlex™ (V8.0) software was used for this analysis. Cytokine levels were measured in duplicate and averaged. Cytokine levels below minimum detectable concentration (MDC) were included with MDCs. The MDC for the specific analytes are as follows (MDC + 2 × Standard Deviation):

Analyte	Sensitivity in serum (pg/ml)
IL-6	1.5 + 0.7
IL-8	2.0 + 0.5
IL-10	2.0 + 0.5
IL-17A	0.5 + 0.1
IL-18	1.3 + 0.9
MCP-1	1.1 + 1.2
TNF-α	0.9 + 0.8

Coefficient of variability (CV) of intra-assay precision for all analytes is <4% and CV of inter-assay precision for the analytes are as follows:

Analyte	CV
IL-6	<21%
IL-8	<17%
IL-10	<13%
IL-17A	<22%
IL-18	<8%
MCP-1	<9%
TNF-α	<14%

2.5 | Histology

We prepared 7-μm paraffin sections which were fixed in Formalin before deparaffinization and rehydration steps were follow. After that the paraffin-embedded sections were stained with the Masson's trichrome kit (MGT) (#3459; Carl Roth GmbH, 192 Karlsruhe, Germany) according to manufacturer's protocol, and counterstained with haematoxylin and eosin for histopathological examinations using MGT the following stains occur: nuclei was stained in black, muscle cells were stained in red/brown, erythrocytes were stained in orange, and connective tissue was stained in green. The sections were analysed with a Zeiss Axiophot microscope and photographed using a digital camera (DS-U1, Nikon) with NIS-Elements BR 3.0 software.

2.6 | RNA extraction, cDNA synthesis, and quantitative PCR

We use E.Z.N.A Total RNA Kit (Omega Bio-tek, Norcross, GA, USA) to isolate total RNA (1 μg) from placental chorionic villous tissue and decidua basalis tissue according to the manufacturer's protocol. Gene expression of B7-H4, TNF-α, IL-6, IL-10, IL-18, IL-α, IL-β were quantitated using the qPCR Master Mix SYBR Green (Affymetrix, Santa Clara, USA) and analyzed using an ABI Prism 7300 sequence detector (Applied Biosystems, Foster City, USA). For details, please refer to our previous publication.[36] As a internal reference HPRT1 (hypoxanthine phosphoribosyltransferase 1) was used. For a detailed description of the PCR parameters, refer to our previous paper.[36] Ten-fold dilutions of purified PCR products were used as standards (B7-H4/IL-10 start from at 100 fg to 0.01 fg, HPRT1 /IL-18/IL-6/IL-1β/TNF-α/ start from 10 fg to 0.001 fg). The quantity of cDNA in each sample was normalized to HPRT1 cDNA. The following experimental groups were tested: pregnant control group ($n = 8$) and COVID-19 affected pregnant group ($n = 8$). The used primers and sequences are listed in Table S1.

2.7 | Western blot

Protein extracts from placental tissues were isolated as described previously.[36] 20 μg protein samples were used for preparing the immunoblot. For specific experimental steps, please refer to our published article.[27] To sum up, for incubation overnight at 4°C the primary antibodies polyclonal rabbit anti-B7-H4 (R&D Systems, #2318A; 1:1000) and purified polyclonal rabbit anti-NF-kb (Santa Cruz, sc-372; 1:250) and for 1 h at room temperature the primary antibody mouse monoclonal-β-Actin Peroxidase (Sigma A3845; 1:200.000) for normalization purposes. The secondary antibody (goat anti-rabbit IgG [H+L], G21234; 1:7500) was used for detecting B7-H4 and NF-kb 1 h at room temperature. Detection was performed with the SuperSignal West Dura Extended Duration Substrate Kit (Thermo Fisher Scientific) according to the protocol. The analysis and quantification was done with the Chemidoc XRS+imaging system (BioRad) and the Image J2× software (Rawak Software Inc.). For normalization purposes, each signal's value was normalized to a same "internal control" sample, which was run on each blot.

2.8 | Immunofluorescence staining

Formalin-fixed paraffin-embedded (FFPE) specimen of placentas were obtained from pregnant patients. FFPE tissue sections (7-μm) were used to perform the immunofluorescence stainings (see details in Duan et al. 2021).[27] The primary antibodies polyclonal rabbit anti-B7-H4 (R&D Systems, #2318A; 1:30), purified polyclonal rabbit anti-NF-kb (Santa Cruz, sc-372; 1:25) were used overnight at 4°C. After that the following secondary antibody was applied: anti-rabbit Alexa Fluor 488 (Life Technologies, A10521; 1:200) and incubated for 1 h at room temperature. Then DNA-specific dye 4′, 6-diamidin-2-phenylindol dihydrochloride (DAPI, 1 μg/ml, Sigma Aldrich) was used to counterstain the nuclei for 15 min at room temperature. Controls were performed by omitting the primary antibody. Examination of stained sections were done by confocal fluorescence microscope (Leica SP5, Leica LAS). For each group, at least four placental tissue samples were used for each experimental group.

2.9 | Statistical analysis

For this study, the sample size was determined by G Power software.

GraphPad Prism 9.2 (GraphPad Software Inc.) was implemented to analyze the data. Outliers were detected by using the Grubbs test. The Mann-Whitney test was used for non-parametric independent two-group comparisons. Observations with missing data were excluded from the relevant analyses.

Data were presented as either median (IQR) or mean ± standard deviation. For all statistical tests, a probability value (p-value) of 0.05 or less was indicated with $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

3 | RESULTS

3.1 | sB7-H4 levels in maternal blood serum/cord blood were increased in SARS-CoV-2 infected pregnant women compared to non-infected pregnant women

It is known that the secretion of sB7-H4 is increased by cells under inflammatory conditions.[28] Here we aim to explore whether the expression of sB7-H4 changed in maternal blood serum from pregnant women with COVID-19 compared to non-SARS-CoV-2 infected pregnant women.

Table 1 displayed the clinical characteristics from women in the third trimester with and without COVID-19 disease.

Maternal age of the reported cases ranged from 26 to 41 years. The length of time from positive SARS-CoV-2 detection to the delivery range from 0 to 14 days. In most cases (70.6%) infections were asymptomatic or with mild symptoms. 29.4% of pregnant woman revealed severe symptoms of pneumonia and dyspnea at the time of diagnosis. The leukocytes in peripheral blood were lower in the COVID-19-affected group compared to the control group. Although the gestational age of delivery in the COVID-19-affected group was significantly earlier in time than in control group, there was no significant difference in newborns birthweight between the two groups. The neonates did not show COVID-19 symptoms and were all tested negative for Sars-Cov-2.

sB7-H4 level was higher in blood serum of pregnant women with COVID-19 compared to the control group ($p = 0.0001288$) (Figure 1A). Comparing the subgroup of women with asymptomatic/mild COVID-19 with the women with severe disease, sB7-H4 blood serum levels were increased in severe diseased women ($p = 0.04557$) (Figure 1B).

TABLE 1 Clinical features of pregnant women and neonatal outcome with and without Sars-Cov2 infection and COVID-19 disease

Variable		COVID-19 ($n = 17$)	Control, non-COVID-19 ($n = 18$)	p-Value
Age, years, mean ± SD		31.94 ± 7.318	32.33 ± 5.541	0.9805
Gestational age at sampling, days, median (IQR)		252.0 (242.5-265)	241.0 (238-246)	0.0188
Gestational age at delivery, days, median (IQR)		267.0 (264-274)	274.5 (270.5-283)	0.0325
Caesarean sections, *n* (%)		7 (41.18%)	9 (50 %)	
BMI (kg/m^2), mean ± SD		29.57 ± 7.684	29.22 ± 5.047	0.7906
Signs and symptoms of mother	Pneumonia, *n* (%)	2 (11.76%)	0	
	Fever, *n* (%)	3 (17.65%)	0	
	Cough, *n* (%)	6 (35.29%)	0	
	Sore throat, *n* (%)	3 (17.65%)	0	
	Dyspnea, *n* (%)	5 (29.41%)	0	
	Myalgia, *n* (%)	1 (5.886%)	0	
	Fatigue, *n* (%)	3 (17.65%)	0	
	Anosmia, *n* (%)	1 (5.88%)	0	
	Ageusia, *n* (%)	1 (5.88%)	0	
	Headache, *n* (%)	1 (5.88%)	0	
Gestational diabetes		3 (17.65%)	0	
Diabetes mellitus		1 (5.88%)	0	
Newborn birthweight (g) mean ± SD		3160 ± 451.0	3499 ± 297.9	0.828
Number of peripheral blood cells, mean ± SD	Hemoglobin	12.04 ± 1.278	11.08 ± 2.095	0.6418
	Leukocyte	7.787 ± 2.465	13.24 ± 2.455	0.0036
	CRP	3.175 ± 4.180	0.700 ± 0.173	0.6066

Abbreviation: BMI, body mass index; COVID-19, coronavirus disease 2019; CRP, C-reactive protein; IQR, interquartile range; SD, standard deviation.

This suggests that the amount of of sB7-H4 correlated with the severity of COVID-19 disease.

sB7-H4 level in cord blood of neonates in case of mothers tested SARS-CoV-2 positive ($n = 8$) was higher compared to control group without maternal infection ($n = 8$) ($p = 0.049$) (Figure 1C). Table S2 displays the neonatal clinical characteristics and outcome from neonates with maternal SARS-CoV-2 infection or without COVID-19. No significant differences in the gestational age, neonatal outcome, newborn birth weight and neonatal arterial pH was observed between the two groups (see Table 1). All newborns were tested negative for SARS-CoV-2 infection.

3.2 | Serum levels of cytokines IL-6 and IL-10 are higher in pregnant women with SARS-CoV-2 infection compared to non-infected pregnancies

Elevated plasma levels of IL-6, IL-10, IL-18, MCP-1, TNF-α, and IL-17A were associated with COVID-19.[16,37,38] Here we examined the expression of these inflammatory factors in serum of pregnant women with and without COVID-19.

Maternal serum of IL-6 and IL-10 levels were significantly elevated in the COVID-19 group compared to the control group ($p = 0.01391$ and $p = 0.02997$) (Figure 2A,B). Other parameters IL-18, MCP-1, TNF-α, and IL-17A showed no differences (Figure 2C–F). Table S3 shows clinical characteristics of patients. The leukocytes in peripheral blood were lower in the COVID-19-affecetd patients than in the control group.

3.3 | Transcript expression of B7-H4 in the placenta villous and decidual basalis tissues of SARS-CoV-2 infected pregnant women compared to non-infected pregnancies

Placenta villous tissue plays a key role in protecting the fetus. Chorionic villous tissues selectively take up and transport various growth factors, nutrients, hormones and cytokines and also transfer passive immunity to the fetus by receptor-mediated transcytosis.[39] We found in the placenta from the SARS-COV-2 infected pregnant women increased areas with fibrinoid necrosis in the periphery of placental villi by Masson's trichrome and Hematoxylen-Eosin staining, which may be caused by the higher inflammation in the SARS-COV-2 infected placenta (Figure S1).

In total, eight placentas of each group (with COVID-19 and without) were analyzed. Table S4 shows the clinical features of pregnant women

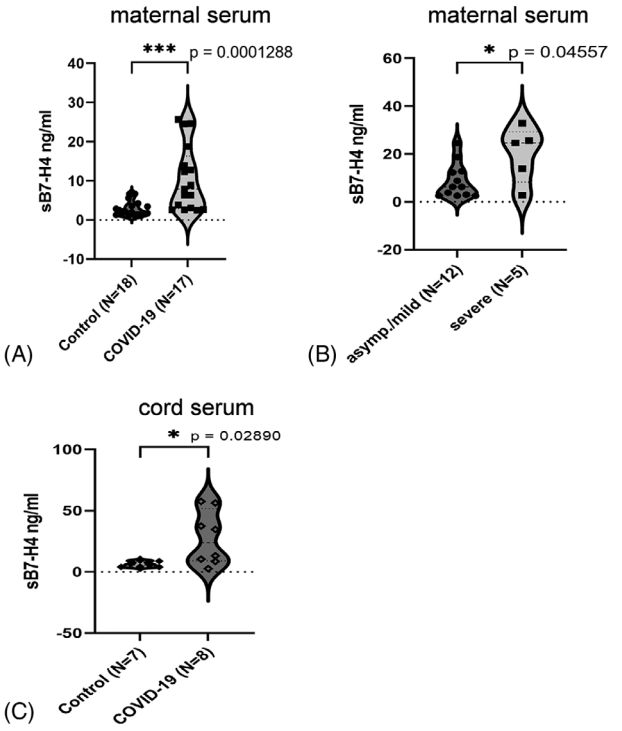

FIGURE 1 sB7-H4 blood serum levels (ng/ml) in the SARS-CoV-2 infected pregnant women (COVID-19) ($n = 17$) and control group ($n = 18$) (A) and sB7-H4 was investigated in the subgroup of women who were screened to be asymptomatic/mild for COVID-19 ($n = 12$) or severe ($n = 5$) (B) Soluble B7-H4 cord blood levels (ng/ml) in neonates with maternal Sars-Cov2 infection (COVID-19) ($n = 8$) and non-infected control group ($n = 7$) (C). Data was represented as means ± SD. $^{*}p < 0.05$, $^{***}p < 0.001$, significantly upregulated compared to control group.

and neonatal outcome with maternal COVID-19 infection or control group. Newborn birthweights were significantly lower in the control group, for other data there were no differences between the groups.

Quantitative RT-PCR was used to detect the mRNA levels of B7-H4 in SARS-CoV-2 infected placentas compared to normal controls. The expression of B7-H4 mRNA was not different in the placenta villous tissue between the COVID-19 positive group and the control group (Figure 3A). In contrast, B7-H4 mRNA showed an increased amount in placenta decidual basalis tissues of COVID-19 patients compared with the controls ($p = 0.0218$, Figure 3B).

3.4 | Transcript expression of cytokine factors IL-6, IL-10, TNF-α, IL-18 and IL-1β in placenta villous/decidual basalis tissues of SARS-CoV-2 infected pregnant women compared to non-infected pregnancies

It is well known that the human placenta is covered by multinucleated STB layer.[40] This forms a physical barrier to vertical infection transmission and is crucial for the interaction between fetal and maternal systemic circulation.[41] Recent publications revealed that many cytokines, such as IL-6, TNF-α, IL-1β, IL-18, and IL-10 were increased in malaria-infected placentas.[41] Here we used quantitative qRT-PCR to analyze the cytokine expression separately in placental chorionic villous tissue and the decidua basalis tissue. We found that only IL-18 and IL-1β were increased in chorionic villous tissue of the COVID-19 positive group compared to controls ($p = 0.0207$ and $p = 0.007$, Figure 4D,E), while other cytokines IL-6/IL-10/TNF-α were not different between the two groups (Figure 4A–C).

Placenta decidual tissue which plays a crucial role in protecting the embryo from attacking by maternal immune cells.[42] Quantitative qRT-PCR was used to detect the cytokine mRNA levels of IL-6, IL-10, TNF-α, IL-18, and IL-1β. Compared with the control group, the mRNA levels of IL-6, IL-10, TNF-α, IL-18, and IL-1β were significantly increased in the placenta decidual tissue of COVID-19 positive group (Figure 4F,H–J). However, IL-10 showed no significantly difference between COVID-19 and the control group (Figure 4G).

3.5 | Protein expression of B7-H4 in the placenta villous and decidual basalis tissues of SARS-CoV-2 infected pregnant women compared to non-infected pregnancies

We performed western blots to analyze also the protein expression of B7-H4 in the placentas of COVID-19 positive pregnant women compared to controls. There was no significant difference analyzing placenta villous tissues (Figure 5A,B). In contrast, in the decidual basalis tissue B7-H4 protein showed higher levels ($p = 0.007$) in the COVID-19 group compared to controls (Figure 5C,D).

In the third trimester, the villous trophoblast consists nearly completely of STB cells by fusion of the cytotrophoblasts (CTBs).[43] Immunolabeling of B7-H4 showed an intense staining at the STB membranes and in the cytoplasm as well as in the mesenchymal stromal cells, weakly staining is found in the nucleus, with no obvious differences in localization and staining intensity between infected and non-infected tissues (Figure 5E,F). The expression pattern in non-infected placentas is similar as previously shown in Duan et al. 2021.[27] B7-H4 was strongly expressed in the decidua basalis tissues of COVID-19 placenta (Figure 5H). However, only few cells revealed a strong B7-H4 expression in control placentas (Figure 5G).

3.6 | Protein level of NF-kb (p65) in the placenta villous and decidual basalis tissues of SARS-CoV-2 infected women compared to the controls

It is known that B7-H4 can inhibit T-cell activation and proliferation by inhibiting NF-kb expression and nuclear translocation.[25] In order to explore whether the B7-H4 expression will affect the NF-kb expression, we analyzed the protein expression of NF-kb (p65) by immunoblotting and immunofluorescence staining.

NF-kb (p65) protein expression was significantly higher expressed in placenta villous tissues ($p = 0.0499$) in the COVID-19 group compared

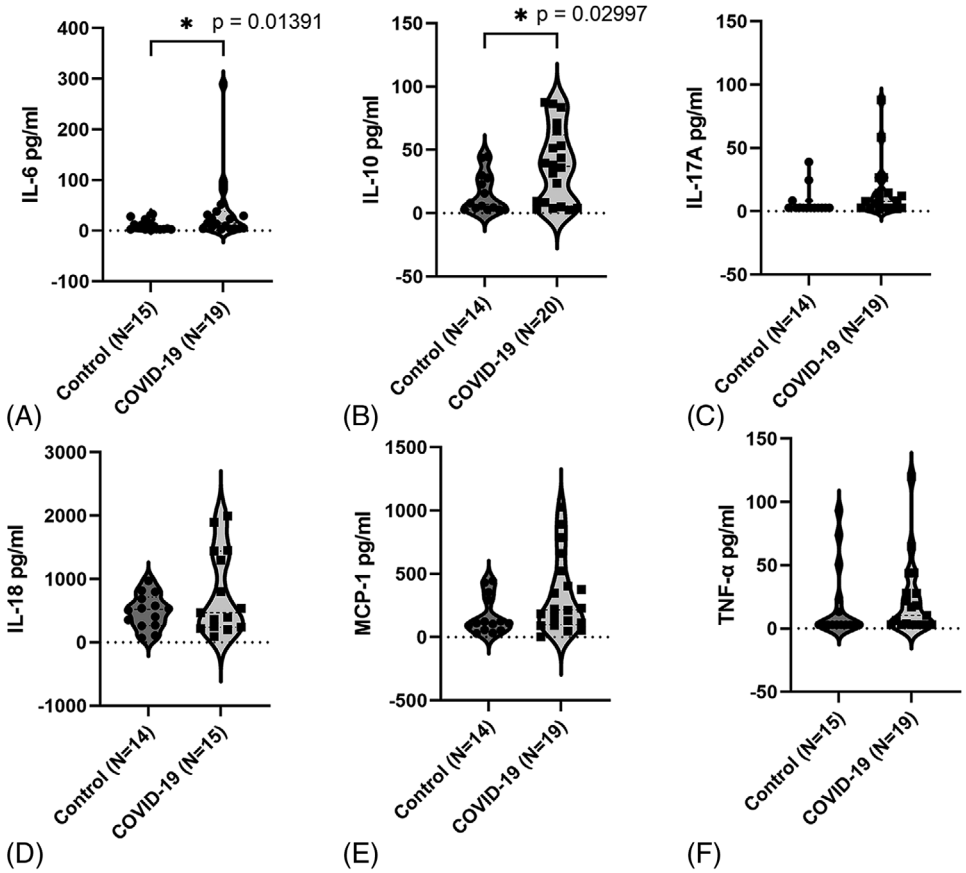

FIGURE 2 IL-6, IL-10, IL-17A, IL-18, MCP-1 and TNF-α levels (ng/ml) in maternal blood sera with Sars-Cov2-infection (COVID-19) ($n = 21$) and non-infected control group ($n = 15$). Data was represented as means ± SD. $^{*}p < 0.05$, $^{**}p < 0.001$ significantly upregulated compared to control group.

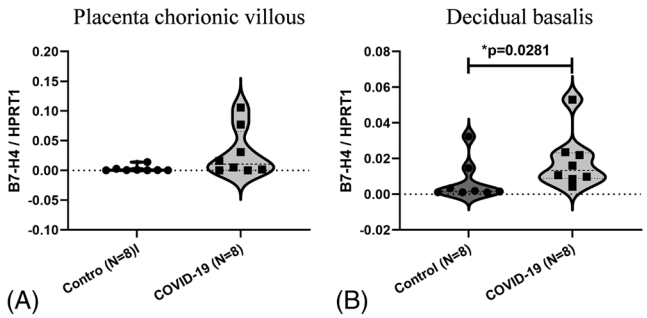

FIGURE 3 Transcript expression of B7-H4 in COVID-19 and control placentas. Results of the B7-H4 mRNA expression in the placenta villous (A) and decidual basalis (B) of COVID-19 ($n = 8$) and control group ($n = 8$). The relative mRNA levels were analyzed by qRT-PCR. The transcript level of B7-H4 was compared after normalization to HPRT1. Data was represented as means ± SD. $^{*}p < 0.05$ significantly up-regulated compared to control group.

to controls (Figure 6A,B). There was no significant differences seen in the decidual tissues between the groups (Figure 6C,D). Immunofluorescence staining of NF-kb (p65) showed an intense staining at the STB membranes and in the cytoplasm and strong staining in the nucleus in the control group (Figure 6E) while the NF-kb (p65) was also stained at the STB membranes and in its cytoplasm and weakly expressed in the nucleus of STB cells of the COVID-19 infected group (Figure 6F). In contrast, in decidua basalis tissue of COVID-19 infected pregnant women and also control placentas, NF-kb (p65) was strongly expressed in the cytoplasm of cells (Figure 6G,H).

4 | DISCUSSION

This research study found that the sB7-H4 level in maternal blood serum and cord blood of COVID-19 affected pregnancies was significantly increased. Interestingly, we found that serum sB7-H4 concentrations correlated with disease severity in subsequent subgroup analyses: severe COVID-19 patients revealed higher sB7-H4 expression levels than patients with mild or asymptomatic infections. According to previous research reports, the secretion of sB7-H4 is increased under inflammatory conditions.[28] Thus, in cases of COVID-19 in pregnancy, pro-inflammatory cytokines are increased in plasma, which may result in the secretion of sB7-H4. In our previous studies, sB7-H4 has been also detected in pregnant women with preterm premature rupture of fetal membranes and preeclampsia as a potential biomarker of immune imbalance.[35,44]

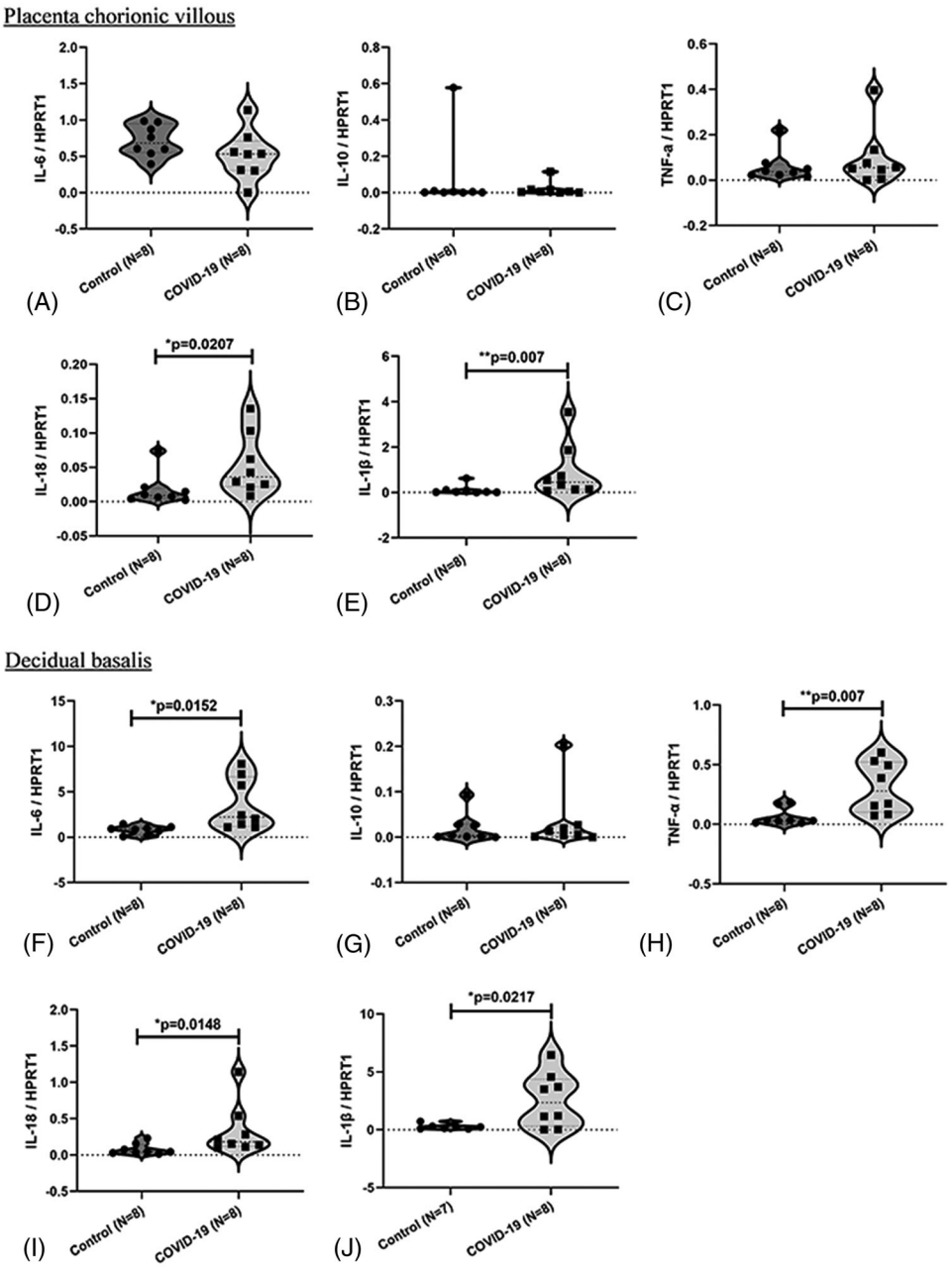

FIGURE 4 Transcript expression of IL-6, IL-10, TNF-α, IL-18, IL-1β (A–E) in the chorionic villous tissues and (F–G) in the decidual basalis tissues of COVID-19 ($n = 8$) and control placentas ($n = 8$). The relative mRNA levels of examined genes were compared after normalization to HPRT1. Data represent means ± SD. $^*p < 0.05$ significantly upregulated compared to controls.

It was reported that IL-10 and IL-6 on monocytes, macrophages, and myeloid dendritic cells can stimulate B7-H4.[45–47] The increased expression of B7-H4 in macrophages was stimulated by IL-6 or IL-10 and showed a stronger ability to inhibit T cell proliferation.[45] In our study we also found that IL-6 and IL-10 were significantly increased in the COVID-19 positive maternal blood serum, which may trigger the elevated expression of sB7-H4 in infected pregnant women.

Lymphocytes play an essential role in the progression of the disease, and most infections with COVID-19 may cause lymphocyte reduction to less than 5% within 2 weeks.[48] A meta-analysis also reported that CD4+ T- and CD8+ T-lymphocytes counts can be used to predict COVID-19 severity.[49] As yet, in vitro and in vivo studies revealed that B7-H4 is a co-inhibitor of CD4+ and CD8+T -cell proliferation, cell-cycle progression as well as anti-tumor immunity.[20–22,50,51] Here we illustrated that sB7-H4 was higher in COVID-19 patients and raised with increased severity of infection. We also found a decrease in leukocytes associated to an increase in sB7-H4 in COVID-19 positive patients, but up to now we did not measure CD4+- and CD8+ T lymphocytes which will be done in future.

The placenta decidua basalis plays an important role in local immunity in pregnancy.[52] Studies showed that maternal decidual immunity can be awakened after viral infection to rapidly clear pathogens and

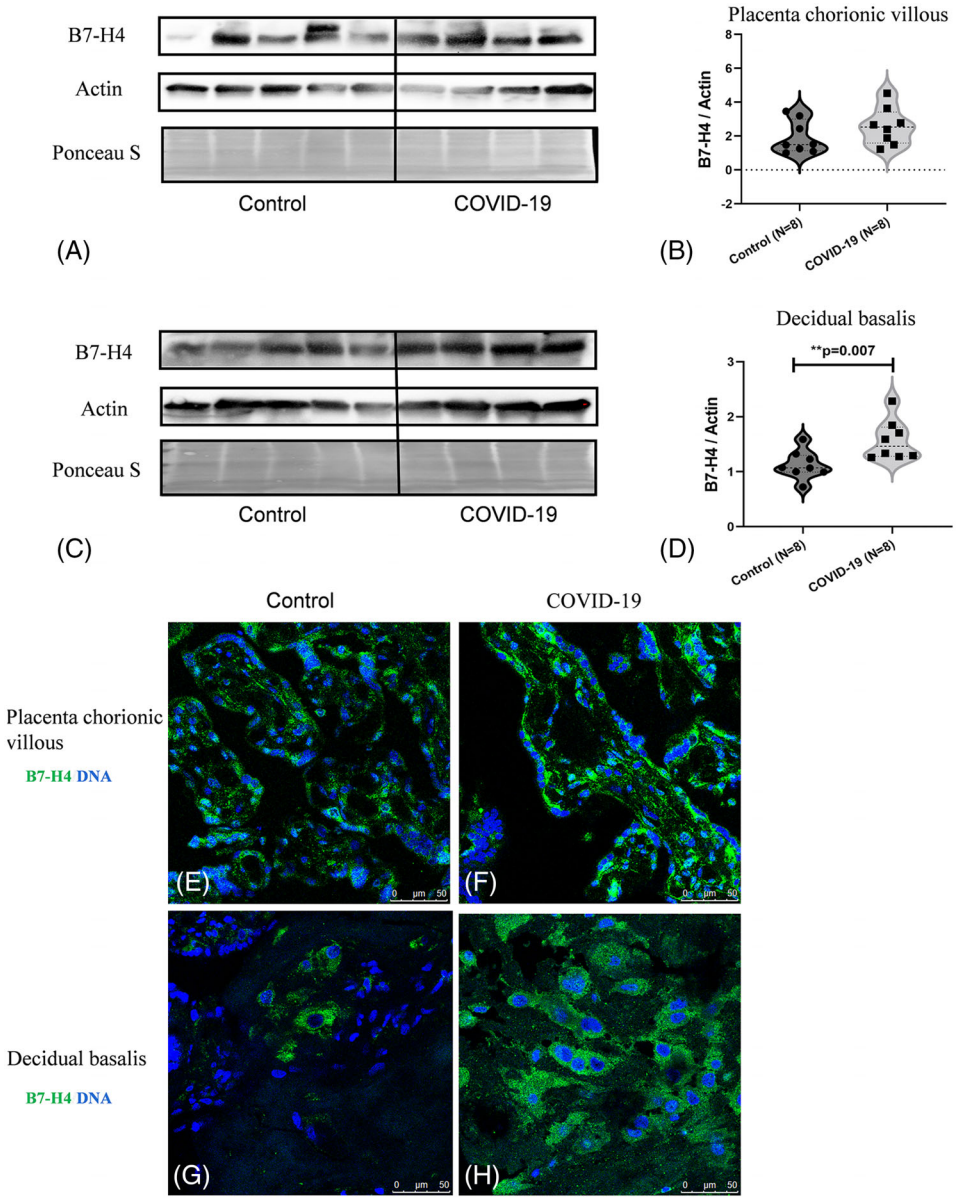

FIGURE 5 B7-H4 protein expression in COVID-19 and control placentas. Analysis of the protein expression of B7-H4 (A–D) in the placenta villous (A, B) and decidual basalis tissues (C, D) of COVID-19 ($n = 8$) and control group ($n = 8$). B7-H4 protein level was normalized to Actin expression and further on normalized to the same sample as internal control which runs on each gel. Ponceau S staining was shown for each blot to show equal amounts of protein loading. Data was represented as means SD. $^{**}p < 0.01$ significantly elevated compared to the control. B7-H4 located in placenta villous tissue (E, F) and decidual basalis tissue (G–H) of non-infected control (E, G) and COVID-19 (F, H) placentas. Green, B7-H4; blue, DAPI, respectively. Scale bar represents: (E–H), 50 μm.

avoid further transmission to the fetus.[53–55] However, the enhanced reactive oxygen species (ROS) production by neutrophils at sites of inflammation leads to endothelial dysfunction and tissue damage.[56] In our study we also found increased fibrotic necrosis of arterioles in placental villi tissue of COVID-19 affected pregnancies, which may cause placental dysfunction.

Recent studies have shown that COVID-19 infection can activate T helper (Th)-1 immunity and cause a significant increase in the level of pro-inflammatory cytokines.[16] Among all inflammatory factors, IL-18 is produced at very early stages of virus infection and it can induce the production of IL-6 and TNF-α, which seems to be a key element for an optimal viral host defense. Previous studies have also reported elevated IL-6 levels in both mild and severe COVID-19 positive patients.[57,58] And the increased secretion of IL-1β subsequently recruits neutrophils to the sites of inflammation, which plays an important role in resisting viral invasion.[59] We found enhanced mRNA levels of IL-6, TNF-α, IL-18, and IL-1β in the decidual tissues of COVID-19-infected women, while for the Th2 immunity cytokine IL-10 there was not different in both groups. As known, increased IL-6 can stimulate B7-H4 expression, whereas B7-H4 can also induce IL-6 expression.[60] Similarly we also found increased expression of B7-H4 in decidua basalis tissue of COVID-19 pregnancies. The increased

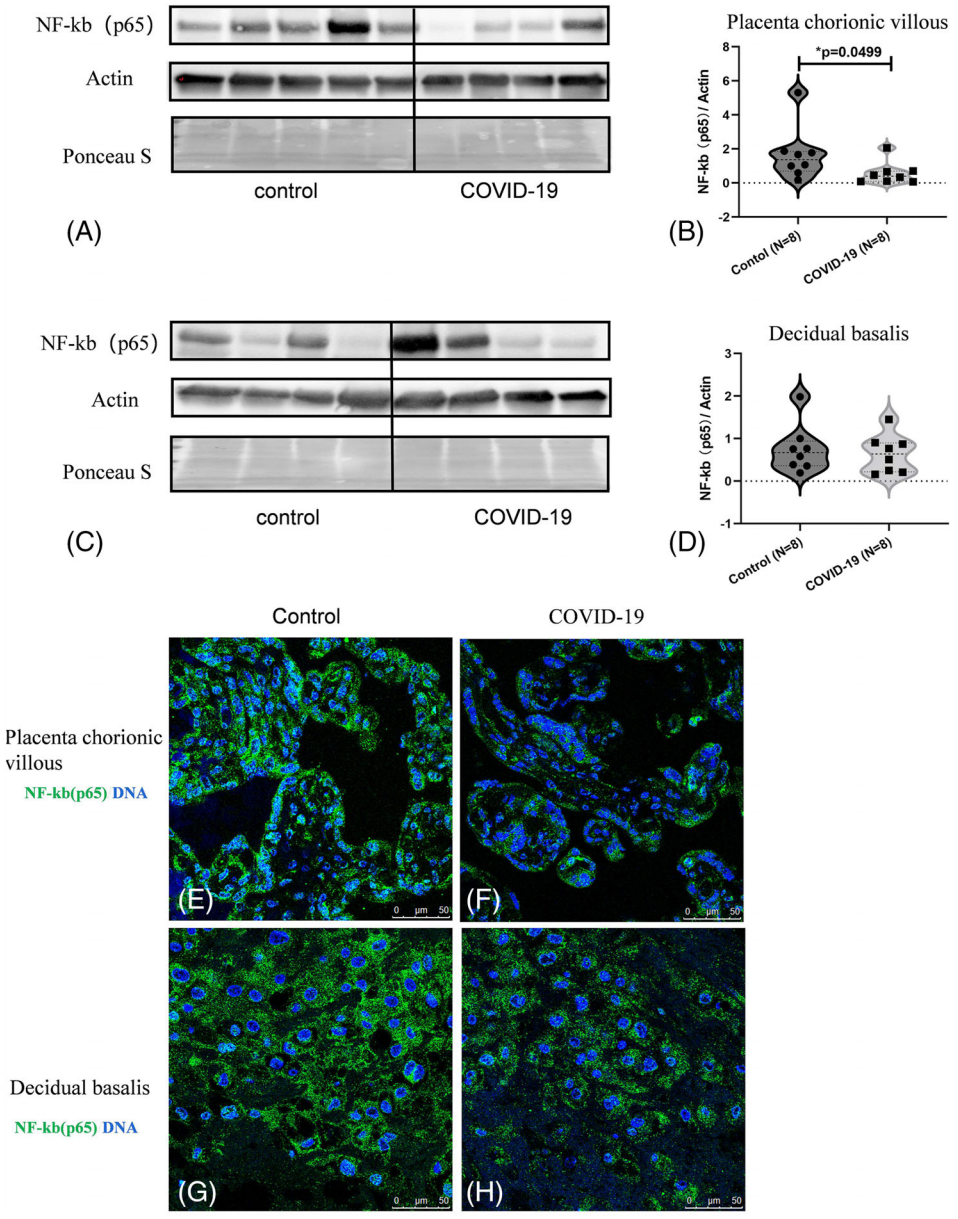

FIGURE 6 (A–D) Protein expression level of NF-kb in the placenta villous and decidual basalis tissues of COVID-19 ($n = 8$) and control group ($n = 8$) by immunoblotting. NF-kb (p65) protein level was normalized to Actin expression and further on normalized to the same sample as internal control which runs on each gel. Data was represented as means ± SD. *$p < 0.05$ significantly downregulated compared to the control. Green, NF-kb (p65); blue, DAPI, respectively. (E–H) Immunolocalization of NF-kb (p65) in placenta villous tissue and decidual basalis tissue (G–H) in COVID-19 placentas (F, H) and non-infected Control placentas (E, G). Scale bar represents: (E–H), 50 μm.

B7-H4 in decidual basalis tissue may inhibit the CD4+ and CD8+T cell proliferation and cell-cycle progression. It is known that CD8+ T-cells contribute to viral control and subsequent elimination. Therefore, in the placental decidua the increase of IL-6 in the inflammatory environment may induce an increased level of B7-H4, which may increase the pro-inflammatory cytokine IL-6 and the generation of lymphocytes, resulting in a relative increase in the production of pro-inflammatory factors and immune imbalance, which contribute to the increased cytokine storm and decreased effectiveness of virus clearance. Like other studies,[14] we found an increase of IL-10 in blood sera of the COVID-19 group compared to the control group. However, Sica et al. showed an elevated expression of B7-H4 inhibited the secretion of IL-10.[20] The results of McElvaney et al.[61] indicate that on the one hand increased pro-inflammatory mediators are associated with severe COVID-19 patients, and on the other hand, loss of protection from anti-inflammatory factors may be clinically relevant. In support of this concept, they showed that the ratios of IL-6:IL-10 were elevated in COVID-19 positive patients admitted to ICU compared to patients with milder symptoms. However, we could not find any difference in the ratio in our cohort.

The placenta villous is in direct contact with maternal blood to ensure nutrient, and gas exchange for the growing fetus. From our

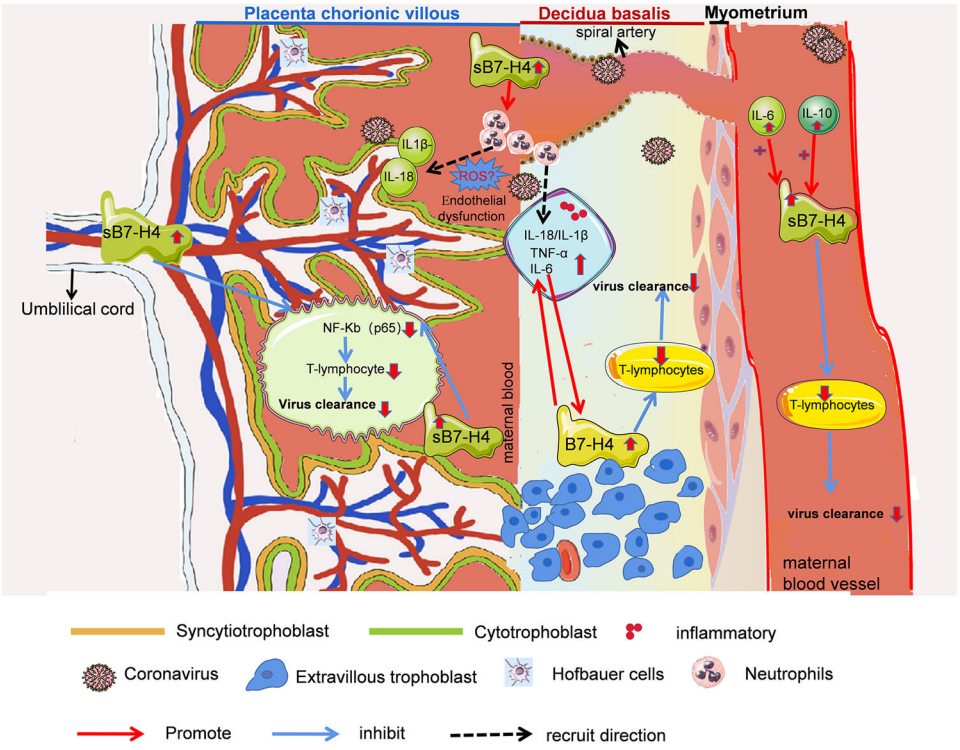

FIGURE 7 Schematic overview of proposed changes in cytokines IL-6 and IL-10 levels in maternal blood serum, cord blood and decidual basalis tissue of COVID-19 affected pregnant women which may cause the increase of sB7-H4 influencing lymphocyte expression and affecting the virus clearance. The increased B7-H4 can also increase the secretion of IL-6. Therefore, this positive feedback loop can increase the secretion of the pro-inflammatory factor IL-6, which may cause a cytokine storm. The increasing expression of sB7-H4 in maternal blood serum and cord blood may affect NF-kb (p65) expression and nuclear translocation in the chronic villous tissue, which may also affect the T-lymphocyte proliferation. sB7-H4 in serum may induce an increase in neutrophils, IL-1β and IL-18 were increased in the placenta villous tissue which will subsequently recruit new neutrophils, leading to fibrinoid necrosis in the periphery of villi and placental endothelial dysfunction.

results of Masson's trichrome and Hematoxylen-Eosin (HE) staining, the fibrinoid necrosis in the periphery of villi was increased in the placentas of patients with COVID-19 compared to the normal control group. Meanwhile, our study showed that IL-6 and B7-H4 in placental villi did not differ between COVID-19 cases and normal pregnant women. Since the expression of sB7-H4 in the maternal blood serum and cord blood is significantly elevated, we speculate that sB7-H4 may affect the placenta villous through blood exchange. Research demonstrated that T cell activation and proliferation could be inhibited by B7-H4 by inducing cell cycle arrest and inhibiting nuclear translocation of NF-kB .[25] Our study showed that NF-kb (p65) was decreased in villous tissues of COVID-19 positive patients compared to controls. The immunofluorescence data let assume that the nuclear expression of NF-kb (p65) seems to be decreased in COVID-19- placenta villous tissues compared to controls. The increased expression of sB7-H4 in maternal blood serum and cord blood may affect the expression of NF-kb (p65), which will inhibit T-cell activation and proliferation. Thus, resulting decrease in CD8+ lymphocytes reduces efficiency of virus clearance. But up to now, we only analyzed the reduction of leukocytes, and we will further study whether B7-H4 will reduce CD4+ and CD8+-lymphocytes. Another study showed that sB7-H4 in serum induced an increase in neutrophils.[62] IL-1β and IL-18 were increased in the placenta villous tissue which will subsequently recruit neutrophils. Therefore, the activity of the immune system is closely related to the progression and severity of COVID-19 disease in pregnant women, and only by understanding its pathogenesis new treatments can be developed.

Limitations of the study are the following: the small cohort of analyzed placentas including only few severe cases. We mostly examined blood samples and placental tissues from pregnant women screened positive for SARS-CoV-2 close to delivery. This fact has the advantage to collect blood samples and placenta tissues of patients with active SARS-CoV-2 infection, however the patients were mostly asymptomatic or showing mild symptoms. We did not perform PCR-testing for SARS-CoV-2 on placental tissues, since it is shown that it is extremely seldom to detect the virus, especially in asymptomatic and mild symptomatic cases.

5 | CONCLUSIONS

sB7-H4 expression levels in maternal blood serum and cord blood has a close relationship with COVID-19 severity which may be related to its crucial role in modulation of the immune system activity during SARS-CoV-2 infection (summarized in the scheme in Figure 7). The increase of IL-6 in the inflammatory environment induces an increase

in the expression of sB7-H4 and B7-H4, which may reduce the proliferation of T-lymphocytes and also induce the pro-inflammatory cytokine secretion accounting for a cytokine storm. Moreover, the increased sB7-H4 may increase the level of neutrophils mediated by elevated levels of IL-1β which recruits more neutrophils to the site of the placenta. These changes may affect placental function due to abnormal placental villi endothelial function resulting in placental dysfunction.

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CONFLICT OF INTEREST

We declare that the research had no commercial or financial relationships that could be constructed as a potential conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available with corresponding author with reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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